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(54) Title: ANGIOGENIC PEPTIDES (57) Abstract The present invention relates to peptides and peptide derivatives related to platelet factor 4 which exhibit angiogenic activi- ty, to pharmaceutical compositions comprising said peptides, and to methods for promoting angiogenesis utilizing said peptides. It is based, in part, on the discovery that an octapeptide derived from platelet factor 4 and seven structurally related peptides (de- picted in Figure 1) were capable of inducing an angiogenic response <i>in vivo</i> as measured by neovascularization in rabbit corneal implant assay and by measurement of capillary endothelial cell chemoattraction. These eight peptides represent specific nonlimit- ing embodiments of the present invention. The angiogenic peptides of the invention may be particularly useful in promoting wound healing, including incisional healing, bone repair, burn healing, and post-infarction repair in myocardial or central ner- vous system injury; and the assimilation of grafted tissues, particularly in persons suffering from vascular insufficiency, such as diabetic patients.		

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-1-
ANGIOGENIC PEPTIDES

1. INTRODUCTION

The present invention relates to peptides and peptide derivatives related to platelet factor 4 which exhibit angiogenic activity, to pharmaceutical compositions comprising said peptides, and to methods of promoting angiogenesis utilizing the peptides of the invention.

2. BACKGROUND OF THE INVENTION

The biological process of neovascularization angiogenesis, the formation of new blood vessels, is necessary for normal development and is also an important aspect of wound repair, and pathological conditions like inflammation, and solid tumor growth (Leibovich et al., 1988, in "Growth Factors and Other Aspects of Wound Healing," Barbul et al., eds., Alan R. Liss, NY p. 132). The angiogenic cascade involves endothelial cell migration, protease production and endothelial cell proliferation (Leibovich et al., supra). Many well known and variously characterized autocrine and paracrine growth factors are involved in the angiogenic cascade. One of the lesser characterized factors is Platelet Derived Angiogenesis Factor (PDAF; Banda et al., 1982, Proc. Natl. Acad. Sci. 79:7773-7777).

2.1. ANGIOGENIC PEPTIDES

Several protein growth factors have been identified which induce angiogenesis. One of the best characterized angiogenic factors is basic fibroblast growth factor, a heparin-binding polypeptide mitogen. Basic fibroblast growth factor (bFGF) proteins have a molecular weight of about 18 kDa, consistent with the predicted cDNA translation product of 155 amino acids, although higher molecular weight forms have also been identified (Sommer et al., 1989, Biochem. Biophys.

Res. Commun. 160:1267-1274; Abraham et al., 1986, EMBO J. 5:2523-2528)

A number of other factors have been reported to exhibit angiogenic activity, including
5 ceruloplasmin (Chu and Olden, 1985, Biochem. Biophys. Res. Commun. 126:15-24); monocyte derived monocytoangiostatin (Wissler et al., 1983, Fed. Proc. 42, Abstract 684); placental angiogenic factor (Burgas, 1986, Eur. J. Clin. Invest. 16:486-493);
10 glioma-derived endothelial cell growth factor (Libermann et al., 1987, EMBO J. 6:1627-1632); and a heparin-binding growth factor from adenocarcinoma of the kidney which is immunologically related to bFGF. A brief review of inflammation and angiogenesis may be
15 found in Folkman et al., 1989, in "Progress in Immunology," Volume VII, Melchers, ed., Springer-Verlag, N.Y., pp. 761-764.

2.2. PLATELET FACTOR 4

20 Platelet factor 4 (PF4), a 70 amino acid heparin-binding protein, is released from the alpha granules of activated platelets. The exact biological function of PF4 is not known, although PF4 is a member of a multigene family involved in chemotaxis,
25 coagulation, inflammation, and cell growth (Eisman et al., 1990, Blood 76:336-344). The genomic sequence of the PF4 gene, and a highly homologous gene, PF4 alt, has recently been reported (Eismann et al., supra). Among the reported biological activities of PF4 are
30 alleviation of concanavalin A-induced immunosuppression in mice (Zucker et al., 1989, Proc. Natl. Acad. Sci. 86:7571-7574); the ability to bind to and enter endothelial cells (Rybak et al., 1989, Blood 73:1534-1539); the elicitation of neutrophil
35 chemotaxis, lysosomal enzyme release and increased

adherence (Bebawy et al., 1986, J. Leukocyte Biol. 39:423-434); stimulation of migration of pericytes but not of smooth muscle cells nor endothelial cells (Bernstein et al., 1982, J. Cell. Sci. (56); 71-82);
5 and a potential anti-thrombotic effect (Weerasinghe et al., 1984, Thromb. Res. 33:625-632). Increased levels of PF4 have been identified in diabetic patients (Guastamacchia et al., 1985, Boll. Soc. Ital. Biol. Sper. 61:499-502; Cortellaro et al., 1990, Thromb.
10 Res. 58:571-576; Cella et al., 1986, Folia Haematol. 113:646-654) and in patients with Behcet's disease (Schmitz-Huebner and Knap, 1984, Thromb. Res. 34:277-286).

15 3. SUMMARY OF THE INVENTION

The present invention relates to peptides and peptide derivatives related to platelet factor 4 which exhibit angiogenic activity, to pharmaceutical compositions comprising said peptides, and to methods
20 for promoting angiogenesis utilizing said peptides. It is based, in part, on the discovery that an octapeptide derived from platelet factor 4 and seven structurally related peptides (depicted in FIGURE 1) were capable of inducing an angiogenic response in
25 vivo as measured by neovascularization in rabbit corneal implant assay and by measurement of capillary endothelial cell chemoattraction. These eight peptides represent specific nonlimiting embodiments of the present invention.

30 The angiogenic peptides of the invention may be particularly useful in promoting wound healing, including incisional healing, bone repair, burn healing, and post-infarction repair in myocardial or central nervous system injury; and the assimilation
35 of grafted tissues, particularly in persons suffering

from vascular insufficiency, such as diabetic patients.

4. DESCRIPTION OF THE FIGURES

5 FIGURE 1. Amino acid sequences of angiogenic peptides Wohl-1 through Wohl-8.

FIGURE 2. Amino acid sequence of PF4.

FIGURE 3. Bar graph showing angiogenic activities of Wohl 1-8 peptides (corresponding to P1-P8) compared to
10 negative and positive (platelet derived angiogenic factor) controls.

FIGURE 4. Diagram for scoring capillary growth toward pellet implant.

FIGURE 5(A-B). Bar graph showing wound healing
15 activity of P-1 (0 μ g/ml, 10 μ g/ml and 30 μ g/ml) at 7 days (5A); and 14 days (5B).

FIGURE 6. Bar graph showing wound healing activity of P-1 (0 μ g/ml, 10 μ g/ml, 30 μ g/ml, and 100 μ g/ml) at 14 days.

20

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the present invention is presented in the following

25 subsections:

- (i) preparation of platelet factor 4;
- (ii) peptides of the invention and their preparation;
- (iii) identification of angiogenic
30 peptides; and
- (iv) utility of the invention.

5.1. PREPARATION OF PLATELET FACTOR 4

Platelet factor 4 (PF4) may be purified
35 using any method known in the art. In a preferred

embodiment of the invention, PF4 may be purified from thrombin-activated platelet extracts by a modification of the method described by Medici et al. (1989, Thrombos. Res. 54:277-287). The PF4 may be isolated by heparin sepharose affinity chromatography with elution of the factor at 1.7 M NaCl, followed by strong anion exchange chromatography on a polysulfoethyl-aspartamide column eluted with NaCl in the presence of about 15% acetonitrile and finally by separation on a Vydac RPC₄ reverse phase HPLC analytical column eluted with a linear acetonitrile gradient in about 0.1% trifluoroacetic acid (TFA) in water.

5.2. PEPTIDES OF THE INVENTION AND THEIR PREPARATION

The peptides of the invention include any peptide which comprises either (i) at least a four amino acid portion of PF4, the amino acid sequence of which is set forth in FIGURE 2, or a functionally equivalent sequence or (ii) at least a six amino acid sequence which is at least 66% homologous to a portion of PF4 sequence as set forth in FIGURE 2, or a functionally equivalent sequence. Homology is to be construed herein as referring to identity between amino acid residues shared by different peptides; for example, a six amino acid residue peptide which is 66% homologous to a six amino acid fragment of PF4 shares 4 amino acid residues with the PF4 fragment which are not necessarily linked together.

In preferred embodiments of the invention, the peptide or peptide derivative comprises the sequence Thr-Ser-Gln and/or Val-Arg-Pro, and more preferably Thr-Thr-Ser-Gln and/or Val-Arg-Pro-Arg.

The peptides of the invention may also comprise portions which bear little or no homology to

PF4. Furthermore, these peptides may be derivatized by conjugation to other compounds, including, but not limited to, carbohydrate, lipid, phosphate, starch, antibody, Fab, Fab₂, enzyme, amino acid, peptide, or growth factor compounds.

The amino acid sequence of PF4 as set forth in FIGURE 2, or a functionally equivalent sequence, should be construed to mean that the PF4 sequence may be (i) that sequence set forth in FIGURE 2 or (ii) the sequence as set forth in FIGURE 2 but in which certain residues are substituted by functionally equivalent amino acids resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In all cases, the peptides of the invention exhibit angiogenic activity as defined in section 5.3, infra.

The peptides of the invention may be prepared by any method known in the art. For example, and not by way of limitation, the peptides may be synthesized (i) by cleavage from a larger peptide, such as, but not limited to, PF4; (ii) by recombinant

DNA expression methods; and (iii) by chemical synthesis, including solid phase techniques as described by Barany and Merrifield (1980, in "The Peptides" Vol. 2, Gross and Meienhofer, eds., Academic Press, N.Y.).

In a preferred specific embodiment of the invention, tryptic digestion of PF4 may be performed to produce PF4 peptide fragments. For example, lyophilized PF4, prepared as described in section 5.1, may be dissolved in 50 μ l of 0.4 M Na_2CO_3 /8M urea at pH=9 in a microcentrifuge tube. The protein may then be reduced by the addition of about 45 mM dithiothreitol in buffer at pH=8 for about 15 minutes at 50°C. The protein may then be carboxymethylated by addition of 5 μ l of iodoacetic acid in 0.5N NaOH and incubated for about 15 minutes in the dark at room temperature. About 140 μ l of deionized water and 5 μ l of a 1 mM HCl solution of sequencer grade trypsin (at 200 μ g/ml) may then be added and the sample incubated at 37 °C for about 24 hours. The resulting tryptic digest may then be injected into an appropriate reverse phase chromatography column, for example, a Vydac C18 column equilibrated with 2.7 percent acetonitrile/0.1 percent TFA/ H_2O and may be chromatographed at an appropriate flow rate, for example 0.5 ml/min with 1.0 minute fractions collected. The elution program may be, for example, 2.7 percent buffer B(95 percent acetonitrile) in buffer A (0.1 percent TFA in water) for about ten minutes, and a gradient of about 27-95 percent buffer B in 123 minutes. Elution of the peptides may be monitored spectrophotometrically at a wavelength of 210 nm. Preferably, a Beckman System Gold HPLC System may be used for chromatography of both proteins and digests. Using the chromatography protocol set forth

as an example supra, Wohl-1 peptide may be expected to elute as peptide number 4 (see section 6, infra). Peptide fragments of PF4 may, according to the invention, be optionally chemically modified, and may
5 be tested for angiogenic activity as set forth in the next section.

5.3. IDENTIFICATION OF ANGIOGENIC PEPTIDES

Peptides as described supra may be
10 determined to have angiogenic activity using any in vitro or in vivo assay system known in the art to evaluate a factor for angiogenic activity. The term angiogenic activity should be construed herein to refer to an ability to (i) induce the formation of new
15 blood vessels and/or (ii) attract endothelial cells.

In a specific embodiment, peptides may be tested for angiogenic activity using an endothelial cell chemotaxis assay, for example, as described in section 6, infra. According to such a method
20 endothelial cell movement in response to a particular peptide may be measured by detecting migration of endothelial cells into a porous membrane.

For example, endothelial cell migration may be assayed by a method such as that described in Banda
25 et al. (1982, Proc. Natl. Acad. Sci. 79:7773-7777, incorporated by reference in its entirety herein). According to this method, solutions of peptides to be tested may be diluted about 1:10 in Dulbecco's modified Eagle's medium supplemented with 10 percent
30 rabbit platelet-poor plasma serum and placed in the bottom of Boyden blind-well chambers. Then gelatin-coated 10- μ m-pore-diameter polycarbonate filters (such as those available from Nucleopore) may be placed over the test solution and endothelial cells suspended in
35 Dulbecco's modified Eagle's medium plus 10 percent

platelet-poor serum may be added to the top compartment, and the chambers may be incubated for about 7 hours at 37°C. At the end of the incubation, the tops of the filters may be wiped clean, and the
5 filters may be fixed, stained, and evaluated by counting the number of cells that migrated to the bottom side of the filter.

In another embodiment, peptides may be tested for angiogenic activity using an in vivo assay
10 which tests for angiogenesis in vivo in response to a peptide of the invention comprised in an implant. According to a specific embodiment, rabbit corneal implant assay (RCIA) method may be used (Gimbrone et al., 1974, J. Natl. Cancer Instit. 52:413 which is
15 incorporated by reference in its entirety herein). In the RCIA, the peptide to be tested is mixed with an inert vehicle such as hydon, a methacrylate polymer, and then dried. The resulting pellet is then
20 implanted in the cornea of a rabbit 2-3 mm from the superior limbus. If the test peptide is angiogenic, capillary growth may be expected to initiate from the superior limbus and then grow toward the implant. A description of the RCIA method is provided in section 7, infra.

25

5.4. UTILITY OF THE INVENTION

The present invention provides for peptides and peptide derivatives related to PF4 which may be used to promote angiogenesis, and for methods of
30 treating patients that would benefit from increased angiogenesis. The invention provides for methods of inducing angiogenesis in a tissue comprising exposing the tissue to an effective amount of a peptide or peptide derivative related to PF4 which exhibits
35 angiogenic activity. Methods of treatment comprise

the administration of an effective amount of a peptide of the invention to a patient in need of such treatment. Administration of the peptide may be systemic or localized. Methods of administration include, but are not limited to, intravenous, intramuscular, subcutaneous, intranasal, oral, or any other appropriate mode. The peptides of the invention may be administered in any suitable pharmacologic carrier. In certain circumstances, it may be desirable to administer the peptides of the invention comprised in an implant such that sustained release of the peptides may be achieved.

Patients who may benefit from increased angiogenesis include patients who suffer from vascular insufficiency, including arterial as well as venous insufficiency, systemically or in a localized area. Examples include patients who are suffering from diabetes or atherosclerosis or disorders of the microcirculation. Distinct areas which might benefit from treatment with angiogenic peptides include, but are not limited to, the extremities, the heart, and the cerebrovascular system. The angiogenic peptides of the invention may be particularly useful for promoting wound healing in diabetics.

The angiogenic peptides of the invention may also be used to promote wound healing in patients who may or may not suffer from vascular compromise. For example, wound healing may be generally promoted in surgical patients, trauma patients, burn patients, or in patients who have suffered damage to the cardiovascular system, including myocardial infarction, or to the nervous system, including central nervous system injury such as trauma or infarction or peripheral nervous system injury. The peptides of the invention may, for example, be used in

the treatment of spinal cord injuries. The peptides may also be useful in improving the cosmetic appearance resulting from wound healing, for example, in scar revision. The peptides of the invention may be useful in the treatment of acute as well as chronic wounds.

Such angiogenic peptides may also be useful in promoting the incorporation of a grafted piece of tissue by providing the tissue with improved blood perfusion. The present invention therefore also provides for methods for facilitating the assimilation of grafted tissue comprising exposing the grafted tissue to an effective concentration of angiogenic peptide.

The angiogenic peptides of the invention may be used for the treatment of human as well as animal subjects.

The present invention also contemplates the development of peptides structurally related to the angiogenic peptides of the invention which inhibit angiogenesis. Such anti-angiogenic peptides would be useful in the treatment of disorders of increased vascularization or in which it is desirable to limit the blood supply or vascularization, such as malignant tumors, hemangiomas, and endothelial angiomatosis rheumatoid arthritis and psoriasis.

6. EXAMPLE: PREPARATION OF ANGIOGENIC PEPTIDES

6.1. MATERIALS AND METHODS

6.1.1. PREPARATION OF PF4

PF4 was purified from thrombin-activated platelet extracts by a modification of the method described by Medici et al. (1989, Thrombos. Res. 54:277-287). PF4 was isolated by heparin sepharose affinity chromatography with elution of the factor at

1.7M NaCl, followed by strong cation exchange chromatography on a polysulfoethyl-aspartamide column eluted with NaCl in the presence of 15 percent acetonitrile and finally by separation on a Vydac RPC,
5 reverse-phase HPLC analytical column eluted with a linear acetonitrile gradient in 0.1 percent TFA in water.

6.1.2. TRYPTIC DIGESTION OF PF4

10 Tryptic digestion was performed by dissolving lyophilized PF4 in 50 μ l of 0.4M Na_2CO_3 /8M urea pH 9.0, in a microcentrifuge tube. The protein was then reduced by addition of 5 μ l of 45mM DTT in pH 9.0 buffer for 15 minutes at 50°C. The protein was
15 carboxymethylated by addition of 5 μ l of iodoacetic acid in 0.5N NaOH and incubated for 15 minutes in the dark, at room temperature. Finally, 140 μ l deionized water and 5 μ l of 1mM HCl solution of sequencer grade trypsin (200 μ g/ml) was added and the sample incubated
20 for 24 hours at 37°C.

The tryptic digest was injected onto a Vydac C_{18} column equilibrated with 2.7% Acetonitrile/0.1% TFA/ H_2O and was chromatographed at a flow rate of 0.5ml/min with 1.0 minute fractions collected. The
25 elution pattern was as follows: 2.7% buffer B (95% acetonitrile) in buffer A (0.1% TFA in water) for 10 minutes, 2.7%-95% B in 123 minutes (see FIGURE 1). The elution of the peptides was monitored at 210 nm. A Beckman System Gold HPLC System was used for
30 chromatography of both proteins and digests.

6.1.3. ENDOTHELIAL CELL CHEMOTAXIS ASSAY

6.1.3.1. PREPARATION OF CELLS

Rabbit wound capillary endothelial cells (RWCE) were grown to 60-85 percent confluency on 3-4 Primaria (Falcon #3824) 75 cm² flasks. Approximately 20-24 hours before the assay, the media was removed and the flasks were rinsed twice with Ca/Mg-free Hank's Balanced Salt Solution. Then 12-15 ml of 0.1% lactalbumin in MEDIA 199 (Media-Tech) was added to each flask and the cultures were maintained overnight. The next day, the lactalbumin/media was removed from the flasks and the cells were rinsed with 6-10 ml HBSS. The endothelial cells were then removed from the flasks by incubating the cells for 14 minutes at room temperature in an enzyme cocktail consisting of 2 x 10⁵ KU DNAase (approximately 100 mg) and 1 x 10⁵ U collagenase (approximately 335 mg) per liter of HBSS (Ca/Mg free). The cells were then scraped from the bottom of the flask and then washed by centrifugation in 0.2 percent lactalbumin/M199 medium. The number of viable cells were determined prior to the migration assay by trypan blue exclusion.

6.1.3.2. PREPARATION OF FILTERS

Nucleopore polypropylene filters (8.0 μm pores, PVPPF, from Neuro Probe, Inc.) were used in the experiment. One side of the filter was coated with a fibronectin solution (1 μg fibronectin per ml HBSS; 3-4 ml were used to coat each filter by putting the solution in a petri dish and then laying the filter over the fibronectin solution).

6.1.3.3. PREPARATION OF CHAMBERS

A Neuro Probe 48 well chemotaxis chamber was used for the experiment. Test peptides, in volumes of about 26 μl, were added to the wells of the lower chamber. The filter, coated with fibronectin and

prepared as above, was then laid over the bottom wells. The top chamber was then attached, and the endothelial cell suspension (0.75×10^6 cells per ml) was added to most of the wells of the top chamber; the remaining wells were filled with cell-free medium as a control. The chamber was then incubated for 4 hours at 37° C in a 5% CO₂, humid atmosphere.

6.1.3.4. REMOVAL AND WIPING OF FILTER

The filter was removed from the apparatus. If the bottom chamber contained an angiogenic peptide, the cells from the top layer would have migrated into and through the filter. However, non-migrated cells which simply adhered to the filter needed to be removed. Therefore, the portion of the filter which had been in contact with the cell-containing chamber was wet in phosphate buffered saline (PBS) and the cells were cleared from this surface using a wiper blade. The filter, which at that point substantially contained migrated cells, was then dried overnight, stained in Leukostat (Fisher), and the absorbance of portions of the filter were read using a densitometer. Relative increases in the densitometry tracing were indicative of greater cell migration and therefore of angiogenic activity of the test peptide contained in the corresponding lower chamber.

6.2. RESULTS AND DISCUSSION

Peptide 4 was sequenced in a Porton 2090e sequencer after adsorption onto a Porton proprietary peptide support. The sequence of the peptide was: Thr-27.5pm, Thr-23.5pm, Ser-30.0pm, Gln-26.5pm, Val-22.6pm, Arg-10.5pm, Pro-9.2pm, Arg-4.7pm. Amino acid analysis using the Beckman Dansyl Chloride method confirmed the total sequence of the peptide. More

consistent results were observed using the rabbit corneal implant assay system modeled after the work of Gimbrone et al. (1974, J. Natl. Cancer Inst. 52:413-427) and Langer et al. (1976, Nature 263:797-800 (see section 7, infra) than with the capillary endothelial cell chemotaxis assay. Our results yielded +2 and +3 angiogenic responses (on a scale of 1 to 4) in at least six different experiments in addition to the data summarized in FIGURE 3. FIGURE 3 summarized results with the original octapeptide (peptide 1), and with seven peptide analogs that were synthesized by the Merrifield method (Barany and Merrifield (1980), in "The Peptides" Vol. 2, Gross and Meienhofer, eds., Academic Press, N.Y.). The results in FIGURE 3 show clearly that as compared to control implant, all of the peptides induced some angiogenic response, but octapeptide 1 and pentapeptide 6 induced very strong and specific angiogenic responses which were also inflammation-free.

7. EXAMPLE: ANGIOGENIC EFFECTS OF PEPTIDES IN RABBIT CORNEAL IMPLANT STUDY

7.1. MATERIALS AND METHODS

7.1.1. PREPARATION OF IMPLANT

Implant pellets were prepared by mixing a solution of 10 percent hydron, 1 percent polyethylene glycol, and 70 percent ethanol with an equal volume of test peptide (50 μ l hydron solution: 50 μ l peptide solution containing about 5-10 μ g of peptide). The resulting mixture was vigorously vortexed, and 20 μ l aliquots were then placed on a sheet of plastic and desiccated to form dry pellets. Each pellet contained about 1 or 2 μ g of test peptide.

7.1.2. SURGICAL PROCEDURE

The resulting implants were placed in the corneas of rabbits under general anesthesia approximately 2-3 mm from the superior limbus capillary bed. The pellets did not lie closer than 1 mm from the capillary bed.

7.1.3. MONITORING ANGIOGENESIS

The eyes were checked on days 3, 5, and 7 for direct growth of capillaries towards the pellet, and were graded according to Figure 4 (Gimbrone et al. 1974, J. Natl. Cancer Inst. 52:413-427). Photographs were taken of the eyes on day 5 and/or 7 to record capillary growth. On day 7, the animals were sacrificed and the corneas were examined histologically (with hematoxylin and eosin stain) for capillary growth.

7.2. RESULTS AND DISCUSSION

Visual examination of angiogenesis was scored on day 7 from 0 to +4 depending on the extent of capillary migration. The angiogenic index (AI) was calculated by the formula

$$AI = (\text{total score}/n \times 8) \times 100,$$

the score being determined by assigning the following numerical values to observed angiogenesis:

	negative = 0
	+/- = 1
	+1 = 2
30	+2 = 4
	+3 = 6
	+4 = 8

The angiogenic activity of peptides Wohl 1-8 is shown in Table I. Each of the eight peptides was observed to be superior to control implants in the

rabbit corneal implant assay (RCIA), with peptides Wohl-1 and Wohl-6 appearing particularly active.

5 The results of histological evaluation of the angiogenic activity of 1.0 $\mu\text{g}/\text{implant}$ as compared to 2.0 $\mu\text{g}/\text{implant}$ is shown in Table II. The 1.0 $\mu\text{g}/\text{implant}$ was found to exhibit a higher angiogenic index than the 2.0 $\mu\text{g}/\text{implant}$.

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TABLE I.

Visual Angiogenic Activity of Peptides

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	<u>Peptide</u>	<u>Number of Observations</u>	<u>Angiogenic Index</u>
	Wohl-1	24	45
	Wohl-2	8	34
10	Wohl-3	7	32
	Wohl-4	8	23
	Wohl-5	8	36
	Wohl-6	8	42
	Wohl-7	8	31
15	Wohl-8	7	30
	Control Implants	24	17

20

TABLE II.

Histological Angiogenic Activity of C8-1

25

	<u>Peptide</u>	<u>Number of Observations</u>	<u>Angiogenic Index</u>
	1.0 µg/implant	11	84
	2.0 µg/implant	7	59
30	Control Implants	16	31

35

8. EXAMPLE: WOUND HEALING ACTIVITY IN RAT INCISIONAL MODEL

The following experiments demonstrate that
5 administration of a peptide of the invention actively
promotes wound healing in an experimental animal
model, i.e., incisional model in normal rats.

8.1 MATERIALS AND METHODS

10

8.1.2. PREPARATION OF TEST MATERIALS

1.0 ml of bovine collagen (Vitrogen 100,
Collagen Corp., Palo Alto, CA) was placed into a
series of 12 x 75 mm polypropylene tubes and
15 lyophilized for 1 1/2 hours. In one set of
experiments, the collagen vehicle was reconstituted
with 1.0 ml of P-1 peptide at a concentration of 10.0
 $\mu\text{g/ml}$ or 30.0 $\mu\text{g/ml}$ in Dulbecco's phosphate buffered
saline (DPBS) at pH 7.0-7.4. In another series of
20 experiments, the collagen vehicle was reconstituted
with 1.0 ml of P-1 peptide at a concentration of 10
 $\mu\text{g/ml}$, 30.0 $\mu\text{g/ml}$ or 100.0 $\mu\text{g/ml}$ in DPBS at pH 7.0-
7.4. For the controls, the collagen vehicle was
reconstituted with 100 μl of DPBS buffer at pH 7.0-
25 7.4. Aliquots (100 μl) of the appropriate test
materials were prepared and kept on ice until
administered to the experimental animals.

8.1.2. SURGICAL PROCEDURE

30

Normal Sprague Dawleys rats, weighing 300-
350 g, were anesthetized with ether. Their backs were
shaved and sterilized with 70% ETOH and Betadine scrub
solution. Two 6 cm linear incisions were made at
full-thickness, 1.5 cm on either side of the midline.

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On day 0, 100 μ l of the appropriate test material was applied, using a positive displacement pipette, to the edges of one incision. 100 μ l of the control material was applied, in the same fashion, to the other incision. The incisions were coapted with four surgical clips (Autoclips, Clay Adams Division of Bechton Dickinson and Co., Parsippany, N.J.).

The animals were housed and fed in standard fashion. At days 7, 14 or 21 post-surgery, the animals were sacrificed and the breaking or tensile strength of the incisions was measured.

8.1.3 TENSILE STRENGTH MEASUREMENT

The animals were euthanized by ether overdose and the entire dorsal skin was excised. Starting 0.5 cm from the edge of the incision, 1.0 cm increments were marked, leaving 0.5 cm on each end. The 1.0 cm strips were cut out and labeled so that they could be paired with the corresponding strips on the opposite incision. 4-5 strips from each incision were stored in phosphate buffered saline at 4°C until the tensile strength was assessed. Samples were tested within 2-3 hours after the animal was sacrificed.

Samples were tested for wound breaking or tensile strength using an Instron Tensiometer (Model 1011, Instron Corp., Park Ridge, IL) with a 50 kg capacity transducer. Samples were clamped in place and pulled at a speed of 4 mm/min. Data was recorded on a chart recorder at a chart speed of 5.0 cm/min.; sensitivity at 1.0 volts.

8.2 RESULTS AND DISCUSSION

Breaking or tensile strength (in grams) was calculated from the maximal height of the graphs obtained using the tensiometer. Data was then statistically analyzed using a T Test for independent observations and a paired T Test. The results are shown in Figures 5(A and B) and 6.

As shown in Figure 5(A and B), incisional wounds healed more rapidly in animals treated with 3.0 μ g of peptide P-1 per incision when compared with animals treated with 1.0 μ g of peptide P-1 or placebo controls. Tensile strength of wounds was greater when measured at Day 7 and Day 14 post-incision. Maximal stimulation of wound healing was noted on Day 14 when compared to placebo controls.

As shown in Figure 6, peptide P-1 enhanced wound healing when administered at a concentration of 3.0 μ g and 10 μ g per incision. Tensile strength was measured on Day 14 post-incision. A dose of 3.0 μ g per incision appeared most active in improving tensile strength; however, a dose of 10.0 μ g per incision was about as active.

The results obtained clearly demonstrated that peptide P-1 of the present invention is active in enhancing wound healing in the rat incisional model at concentrations comparable to those which would be administered to humans. A single administration of an effective dose of peptide P-1 is sufficiently active to stimulate healing in the rat incisional model. These observations confirm the applicability of peptides of the invention for therapeutic use in pathogenic conditions requiring accelerated healing and/or angiogenesis.

The present invention has been described in detail with particular reference to the above embodiments. It will be understood, however, that the

present invention is not to be limited in scope by the embodiments disclosed which are intended as illustrations of aspects of the invention.

Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various references have been cited herein; these are incorporated by reference, in their entirety.

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WHAT IS CLAIMED IS:

1. A peptide or peptide derivative related to platelet factor 4 which exhibits angiogenic activity.
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2. A peptide or peptide derivative which (i) comprises a four amino acid peptide, the sequence of which is comprised in the sequence presented in FIGURE 2 or a functionally equivalent sequence; and
10 (ii) exhibits angiogenic activity.
3. A peptide or peptide derivative which (i) comprises a six amino acid sequence which is at least 66% homologous to an amino acid sequence
15 comprised in the sequence presented in FIGURE 2 or a functionally equivalent sequence; and (ii) exhibits angiogenic activity.
- 20 4. The peptide or peptide derivative of claim 1, 2, or 3 which comprises the sequence Thr-Ser-Gln.
5. The peptide or peptide derivative of claim 1, 2, or 3 which comprises the sequence Val-Arg-Pro.
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6. The peptide or peptide derivative of claim 1 which has the amino acid sequence Thr-Thr-Ser-Gln-Val-Arg-Pro-Arg or is a derivative thereof.
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7. The peptide or peptide derivative of claim 1 which has the amino acid sequence Val-Lys-Thr-Thr-Ser-Gln-Val-Arg-Pro-Arg or is a derivative thereof.
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8. The peptide or peptide derivative of claim 1 which has the amino acid sequence Ser-Gln-Val-Arg-Pro-Arg or is a derivative thereof.

5 9. The peptide or peptide derivative of claim 1 which has the amino acid sequence Val-Arg-Pro-Arg or is a derivative thereof.

10 10. The peptide or peptide derivative of claim 1 which has the amino acid sequence Thr-Thr-Ser-Gln-Val-Arg-Pro-Arg-His-Ile-Thr or is a derivative thereof.

15 11. The peptide or peptide derivative of claim 1 which has the amino acid sequence Thr-Thr-Ser-Gln-Val or is a derivative thereof.

20 12. The peptide or peptide derivative of claim 1 which has the amino acid sequence Thr-Ser-Gln-Val-Arg or is a derivative thereof.

25 13. The peptide or peptide derivative of claim 1 which has the amino acid sequence Thr-Thr-Ser-Gly-Ile-His-Pro-Lys or is a derivative thereof.

14. A pharmaceutical composition comprising the peptide or peptide derivative of claim 1 in a pharmacologically suitable carrier.

30 15. A pharmaceutical composition comprising the peptide or peptide derivative of claim 2 in a pharmacologically suitable carrier.

16. A pharmaceutical composition comprising the peptide or peptide derivative of claim 3 in a pharmacologically suitable carrier.

5 17. A pharmaceutical composition comprising the peptide or peptide derivative of claim 4 in a pharmacologically suitable carrier.

10 18. A pharmaceutical composition comprising the peptide or peptide derivative of claim 5 in a pharmacologically suitable carrier.

15 19. A pharmaceutical composition comprising the peptide or peptide derivative of claim 6 in a pharmacologically suitable carrier.

20 20. A pharmaceutical composition comprising the peptide or peptide derivative of claim 7 in a pharmacologically suitable carrier.

21. A pharmaceutical composition comprising the peptide or peptide derivative of claim 8 in a pharmacologically suitable carrier.

25 22. A pharmaceutical composition comprising the peptide or peptide derivative of claim 9 in a pharmacologically suitable carrier.

30 23. A pharmaceutical composition comprising the peptide or peptide derivative of claim 10 in a pharmacologically suitable carrier.

35 24. A pharmaceutical composition comprising the peptide or peptide derivative of claim 11 in a pharmacologically suitable carrier.

25. A pharmaceutical composition comprising the peptide or peptide derivative of claim 12 in a pharmacologically suitable carrier.

5 26. A pharmaceutical composition comprising the peptide or peptide derivative of claim 13 in a pharmacologically suitable carrier.

27. A method of inducing angiogenesis in a
10 tissue comprising exposing the tissue to an effective amount of a peptide or peptide derivative related to platelet factor 4 which exhibits angiogenic activity.

28. A method of inducing angiogenesis in a
15 tissue comprising exposing the tissue to an effective amount of a peptide or peptide derivative (i) which comprises a four amino acid peptide, the sequence of which is comprised in the sequence presented in FIGURE 2, or a functionally related sequence; and (ii)
20 exhibits angiogenic activity.

29. A method of inducing angiogenesis in a tissue comprising exposing the tissue to an effective amount of a peptide or peptide derivative which (i)
25 comprises a six amino acid sequence which is at least 66% homologous to an amino acid sequence comprised in the sequence presented in FIGURE 2 or a functionally equivalent sequence; and (ii) exhibits angiogenic activity.
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30. The method according to claim 27, 28,
c 29 in which the peptide or peptide derivative comprises the sequence Thr-Ser-Gln.

31. The method according to claim 27; 28,
or 29 in which the peptide or peptide derivative
comprises the sequence Val-Arg-Pro.

5 32. The method according to claim 27, 28,
or 29 that is performed in vivo.

33. The method according to claim 32 that
is performed in a human patient.

10 34. The method according to claim 33 in
which the patient is suffering from vascular
insufficiency.

15 35. The method according to claim 33 in
which the patient is suffering from diabetes.

36. The method according to claim 27, 28,
or 29 which is used to promote wound healing in a
20 diabetic patient.

37. The method according to claim 27, 28,
or 29 which is used to promote wound healing of
pressure ulcers.

25 38. The method according to claim 27, 28,
or 29 which is used to promote wound healing of
myocardial infarction.

30 39. The method according to claim 27, 28,
or 29 which is used to promote wound healing in
nervous system damage.

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40. The method according to claim 27, 28, or 29 which is used to promote wound healing in burn patients.

5 41. The method according to claim 27, 28, or 29 which is used to promote wound healing in scar revision.

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1	Thr	Thr	Ser	Gln	Val	Arg	Pro	Arg
2	Val	Lys	Thr	Thr	Ser	Gln	Val	Arg
3			Ser	Gln	Val	Arg	Pro	Arg
4					Val	Arg	Pro	Arg
5	Thr	Thr	Ser	Gln	Val	Arg	Pro	Arg
6	Thr	Thr	Ser	Gln	Val			Ile Thr
7			Thr	Ser	Gln	Val	Arg	
8	Thr	Thr	Ser	GLY	Ile	His	Pro	Lys

FIG.1

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	5	10	15	20	25	30																							
M	S	A	A	G	F	C	A	S	R	P	G	L	L	F	L	G	L	L	L	P	L	V	V	A	F	A	S		
A	E	A	E	E	D	G	D	L	Q	C	L	C	V	K	T	T	S	Q	V	R	P	R	H	I	T	S	L	E	V
I	K	A	G	P	H	C	P	T	A	Q	L	I	A	T	L	K	N	G	R	K	I	C	L	D	L	Q	A	P	L
Y	K	K	I	I	K	K	L	L	E	S																			

THREE-LETTER ABBREVIATION	ONE-LETTER SYMBOL
Ala	A
Arg	R
Asn	N
Asp	D
Asx	B
Cys	C
Gln	Q
Glu	E
Glx	Z
Gly	G
His	H
Ile	I
Leu	L
Lys	K
Met	M
Phe	F
Pro	P
Ser	S
Thr	T
Trp	W
Tyr	Y
Val	V

FIG.2

SUBSTITUTE SHEET

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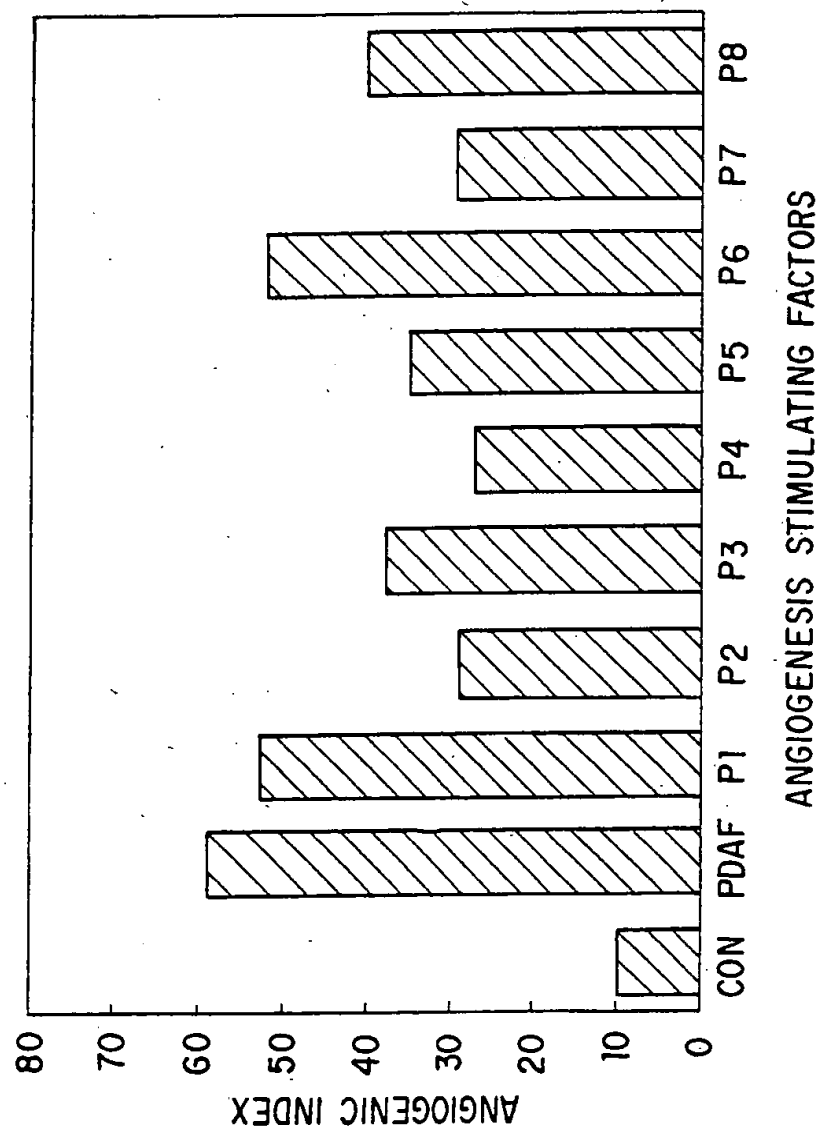


FIG. 3

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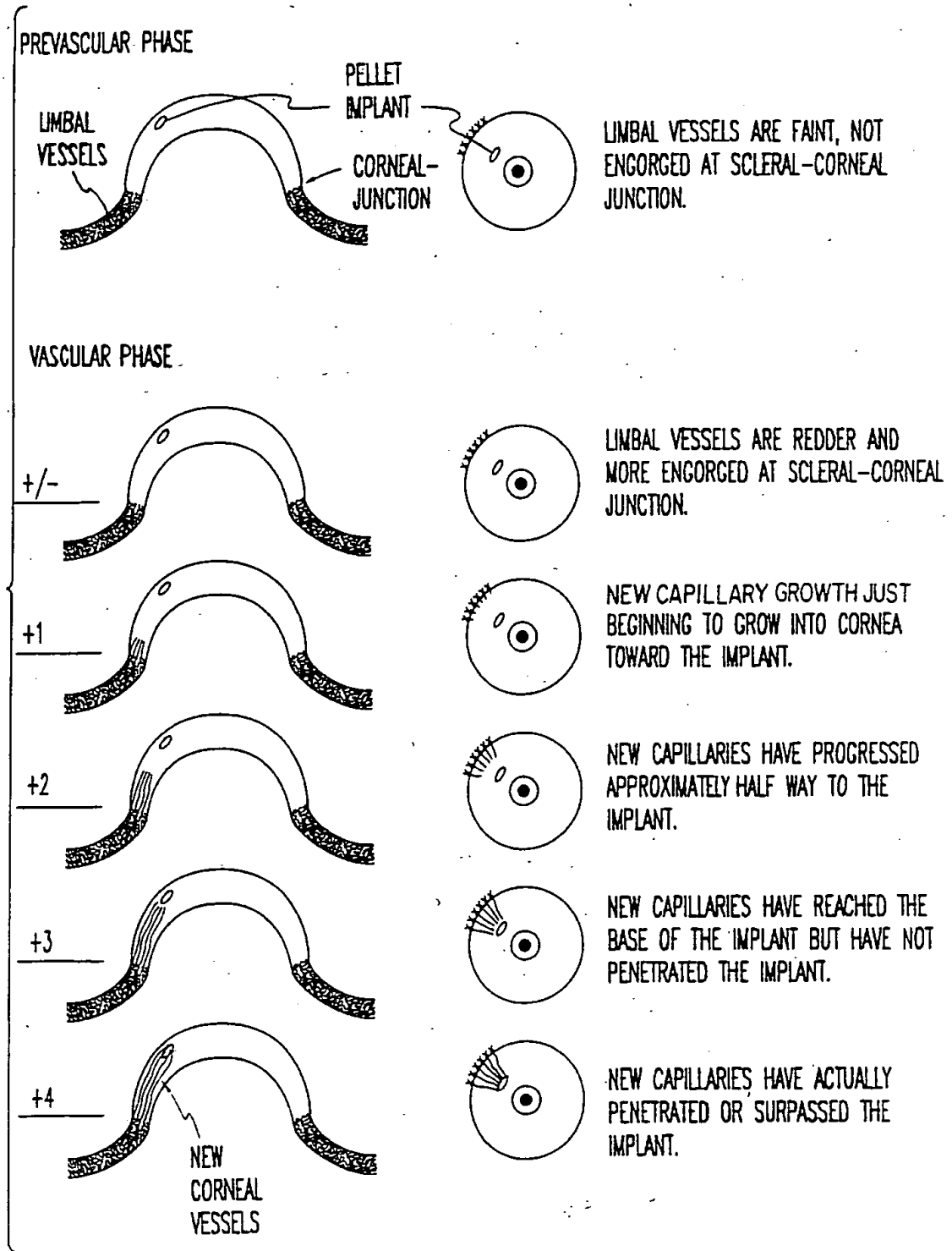


FIG. 4

SUBSTITUTE SHEET

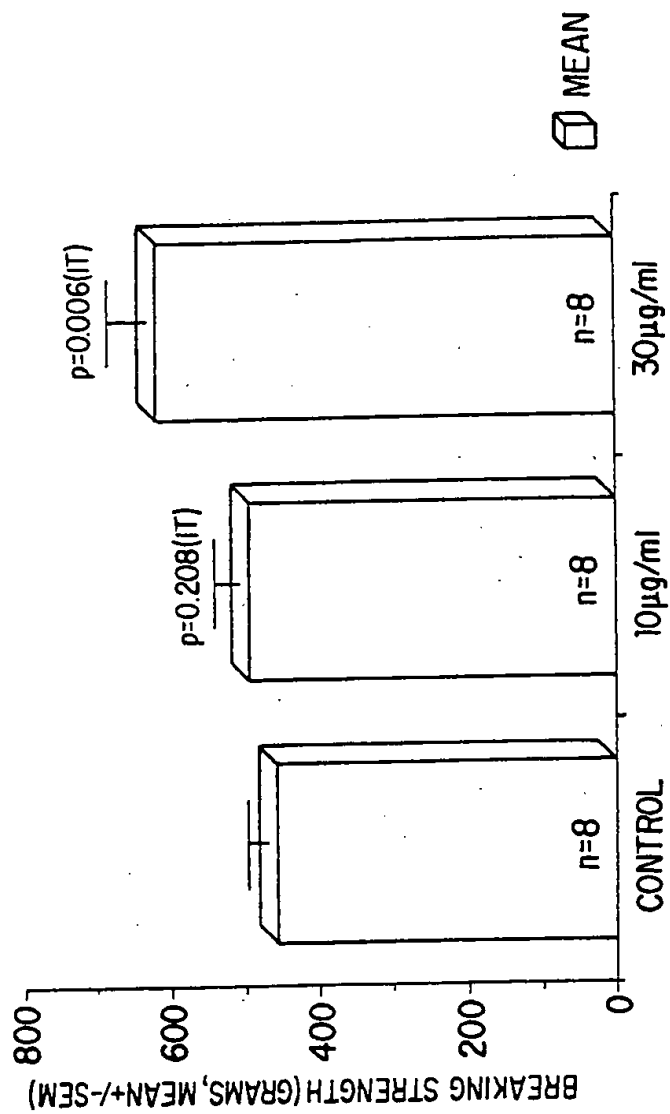
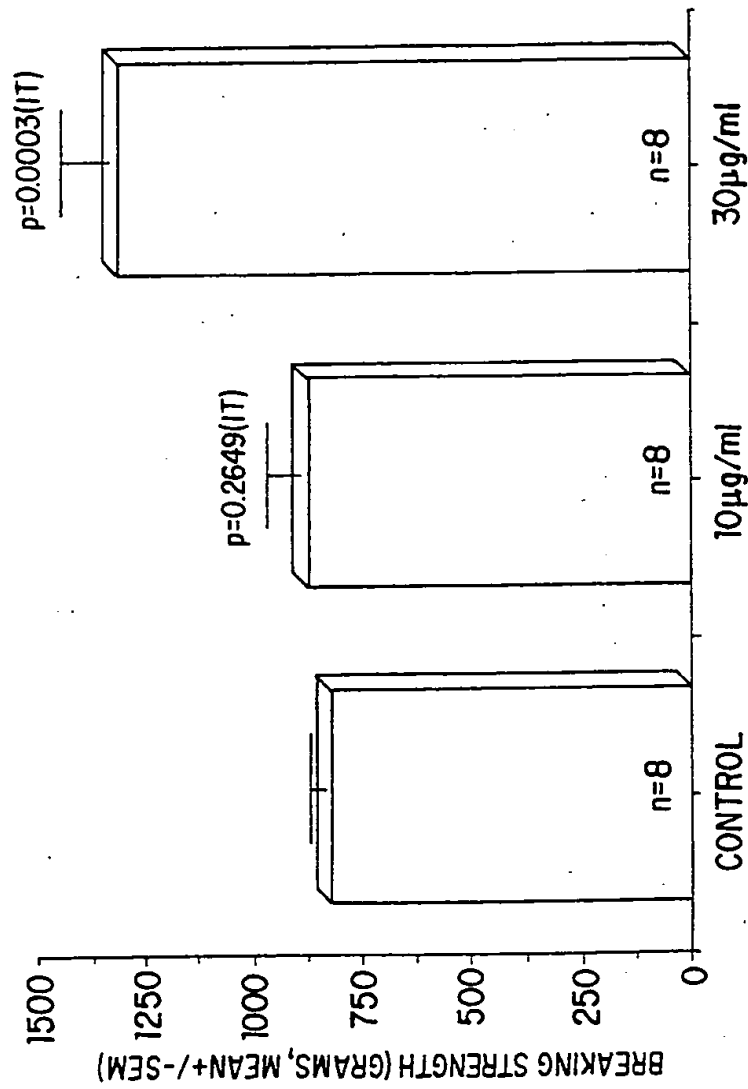


FIG. 5A



DAY 14
FIG. 5B

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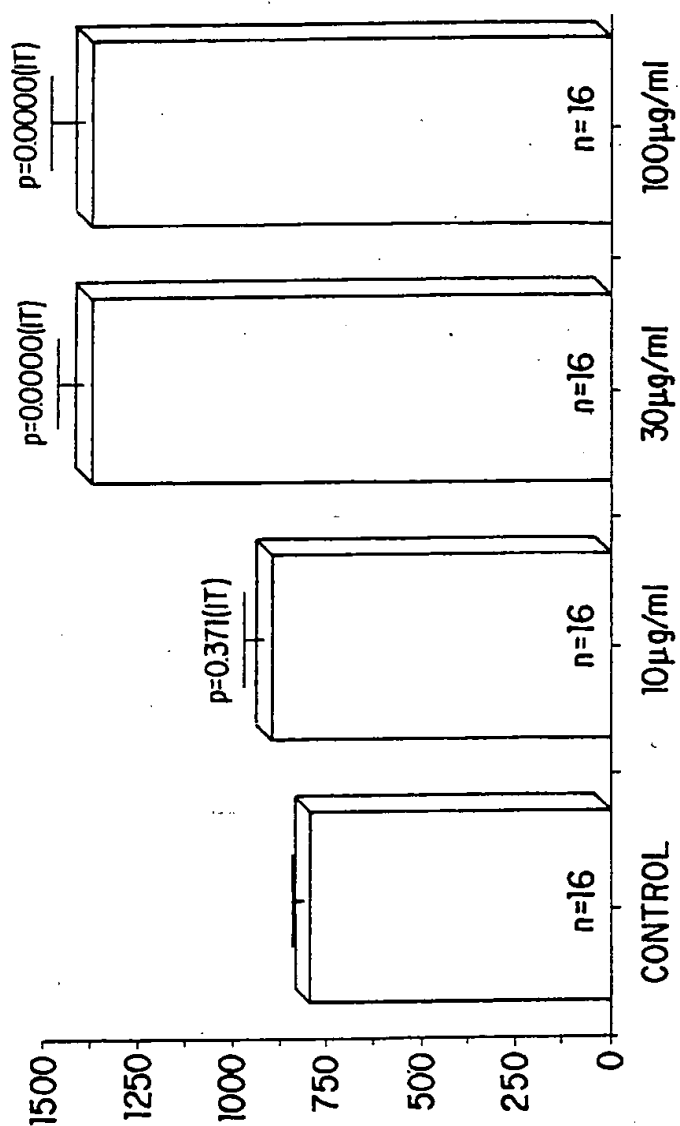


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/09813

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ²		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): A61K 37/00, 37/02 US CL : 514/13; 530/330, 331		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	514/13; 530/330, 331	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
CAS SEQUENCE SEARCH		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ¹	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X/Y	US, A, 4,645,828 (TWARDZIK ET AL.) 24 February 1987. See column 3 and columns 7-9.	1-12, 14-25/27-41
X/Y	EP, A, 0,378,364 (MAIONE ET AL.) 18 July 1990. See claims.	1-12, 14-25, 27-41/27-41
Y	Archives of Biochemistry and Biophysics Vol. 250, No. 1 issued October 1986, CIAGLOWSKI ET AL. "Isolation and Amino Acid Sequence Of Bovine Platelet Factor 4", pages 249-256. See page 254 Figure 3.	13, 26
<p>¹ Special categories of cited documents:¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
06 MARCH 1992	02 APR 1992	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	Bennett Celsa 19 